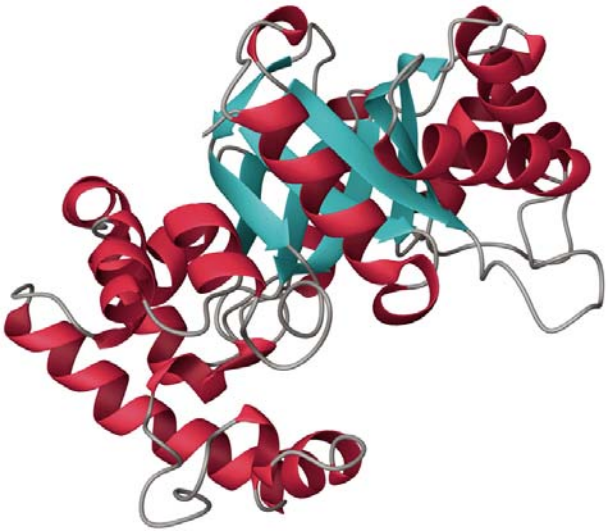
MILLIPORE

hKv3.3-CHO K1 Recombinant Cell Line

cat. #CYL3045

Revision 1



Ordering Information and Technical Services:

MILLIPORE (UK) LIMITED
6-7 Technopark
Cambridge
CB5 8PB
UK
Tel: +44 (0) 1223 508191
Fax: +44 (0) 1223 508198

Customer Services UK: 0800 0190 333
US: 800 437 7500

www.millipore.com/ionchannels

Contents:

Licensing Statement

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242, USA.

Use of IRES is covered by U.S. Patent 4,937,190 and is limited to use solely for research purposes. Any other use of IRES requires a license from Wisconsin Alumni Research Fund (WARF).

The bovine growth hormone (bgh) polyadenylation signal is patented under U.S. Patent No. 5,122,458. Use, in the USA, of the bgh polyadenylation signal found in screening systems sold by Millipore requires a license from Research Corporation Technologies, Inc. (RCT). After purchasing these materials from Millipore, you must contact RCT within 30 days to obtain a commercial license. The bgh polyadenylation signal cannot be used until a commercial license is obtained. Contact Jennifer Caldwell, Ph.D., at Research Corporation Technologies, Inc., 101 North Wilmot Road, Suite 600, Tucson, AZ 85711-3335, USA. Tel: 1-520-748-4400, Fax: 1-520-748-0025.

Product description:
Recombinant CHO-K1 cell line expressing human Kv3.3.

Format:
2 x 1ml vials (1.80 x 10^6 cells/ml) of hKv3.3-CHO K1 at passage 11.

Mycoplasma Testing:
The cell line has been screened using the MycoSensor™ PCR Assay Kit (Stratagene) to confirm the absence of Mycoplasma species.

Functional Validation:
CHO-K1 cells expressing hKv3.3 were characterised in terms of their pharmacological and biophysical properties using whole-cell patch clamp techniques and IonWorks™ HT.

Using whole-cell patch clamp techniques the threshold of current activation was found to occur when the membrane potential was depolarised to potentials more positive than -20 mV, with the current inactivating at potentials greater than 0 mV. The mean current at +40 mV was 11.69 ± 1.06 nA (n = 5).

The values of $V_{½}$ of activation and inactivation were approximately 10 mV and –19 mV respectively, in line with published data.

Currents were inhibited by µM concentrations of the known potassium channel blockers TEA and 4-AP.

The currents obtained with IonWorks™ HT were typical of homomeric hKv3.3 currents since they had an initial fast activating component followed by a slowly inactivating component.

Functional channel expression over time was monitored using IonWorks™ HT. Channel expression is robust over at least 37 passages. At passage 37, 95% of cells sealed with a resistance >50 MOhm. Of these, 100% expressed hKv3.3 currents >500 pA with a mean current amplitude of 4.80 ± 0.10 nA (n=183).

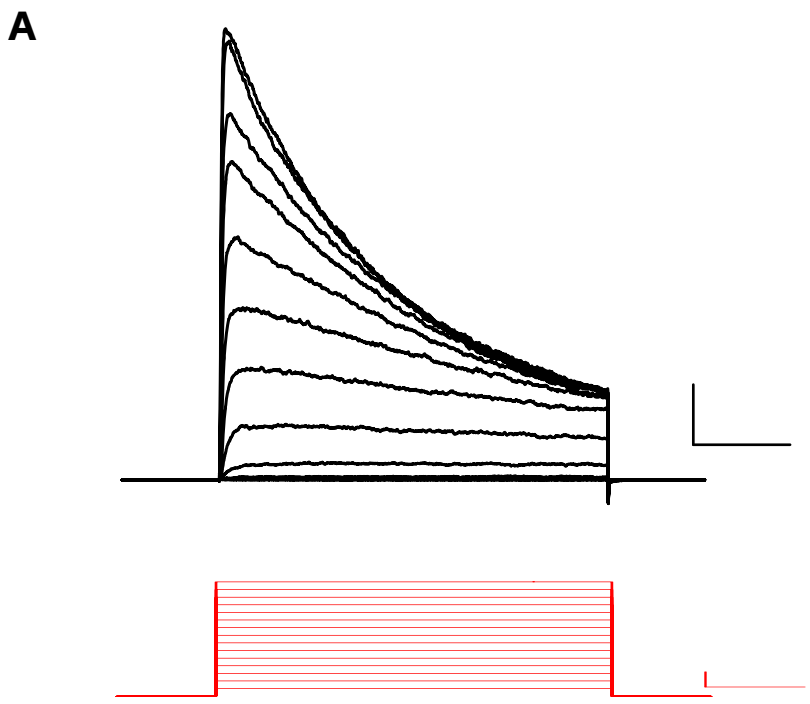
IonWorks™ HT is a trademark of Molecular Devices Corporation

Electrophysiological Properties of the hKv3.3 current.

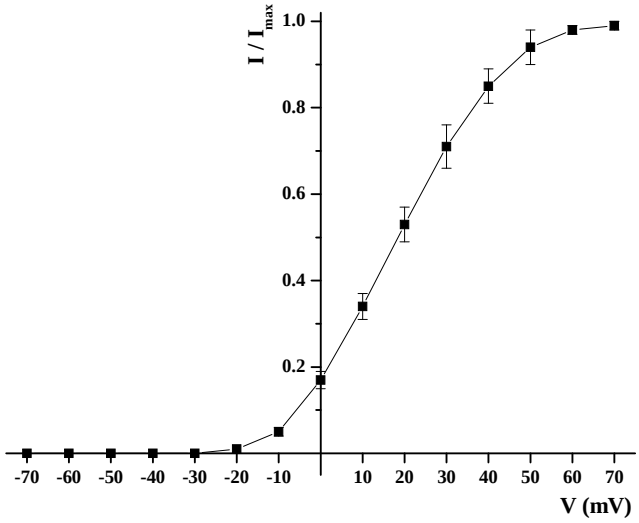
Conventional Whole-Cell Patch Clamp Electrophysiology.

Current / Voltage Relationship:
The Kv3.3 channel is a slow inactivating A-type voltage-gated potassium channel that is found mainly in the brain (Rudy and McBain, 2001; Gutman *et al.*, 2005). The biophysical properties of the hKv3.3 current were studied by stepping from the holding potential of -80 mV to voltages of -70 mV to +70 mV in 10 mV increments, every 10 s. At voltages positive to 0 mV, outward currents noticeably inactivated during the 200 ms step in a voltage dependent manner (**Figure 1A**). The threshold of activation was at potentials slightly more positive than -20 mV (**Figure 1B**). These results are in agreement with previous data where Kv3.3 currents were reported to activate at voltages positive to -30 mV (-30 mV to -10 mV range, Vega-Saenz de Miera *et al.*, 1992; Coetzee *et al.*, 1999; Rudy *et al.*, 1999; Fernandez *et al.*, 2003)

Figure 1. Current / Voltage relationship.
A. Typical current traces (upper panel) elicited by 200 ms depolarising voltage pulses from –70 mV to +70 mV in 10 mV increments (lower panel) from a holding potential of –80 mV. Scale bars represent 50 ms and 2 nA in the upper panel and 50 ms and 20 mV in the lower panel.
B. Mean I/V relationship: Peak current amplitudes were measured during the 200 ms step and plotted against the test voltage. Peak currents at a given voltage were normalized to the peak current amplitude obtained at +60 mV. The mean current at +40 mV was 11.69 ± 1.06 nA (n = 5).



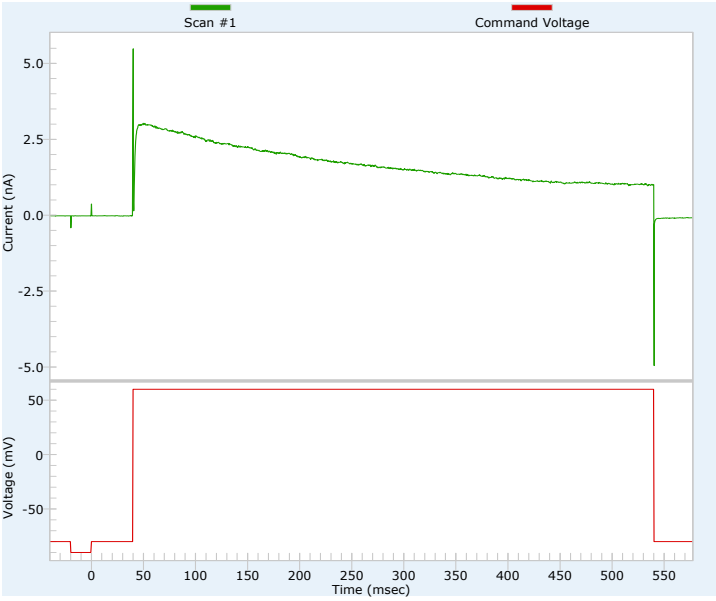
B



IonWorks™ HT Electrophysiology.

To activate the currents the membrane voltage was stepped from a holding potential of -80 mV to +60 mV for 500 ms before returning to the holding potential. A typical current evoked by this voltage protocol is shown in **Figure 2**. This current trace shows all the hallmarks of homomeric hKv3.3 currents, since it has an initial fast activating component followed by a slowly inactivating component.

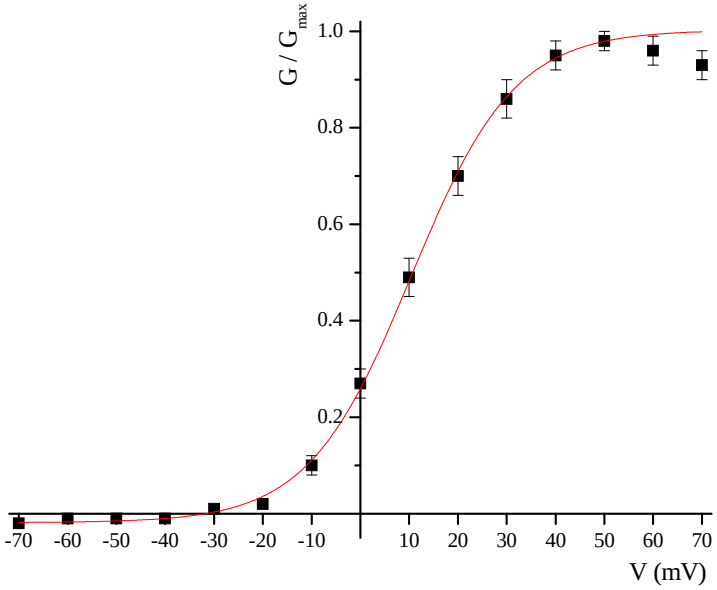
Figure 2. A typical evoked current on IonWorks™ HT. A typical current (green trace) evoked by a voltage step to +60 mV (red trace).



Manual Patch Clamp Electrophysiology.

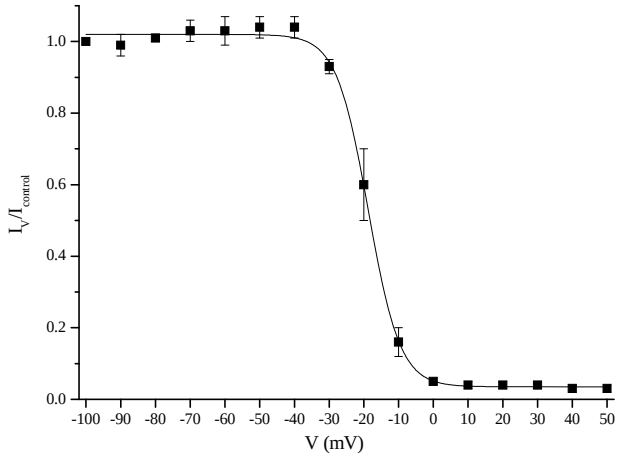
Conductance / Voltage Relationship:
The voltage that produces half maximal activation of the channels ($V_{½}$) was 10.7 ± 2.0 mV and the slope of the conductance-voltage relationship (k) was 10.4 ± 0.9 (**Figure 3**). This result is in agreement with previous findings. $V_{½}$ has been found to range between 3 – 12 mV and k varies between 6 – 14 (Vega-Saenz de Miera *et al.*, 1992; Rudy *et al.*, 1999; Rae and Shepard, 2000; Fernandez *et al.*, 2003; Waters *et al.*, 2006).

Figure 3. Conductance / Voltage relationship.
The conductance was calculated assuming a potassium reversal potential (E_k) of -90 mV. Conductance was normalized to the peak conductance and could be described by a Boltzmann equation. $V_{½}$ = 10.7 ± 2.0 mV; k = 10.4 ± 0.9.



Inactivation:
Using the voltage protocol described in **Figure 4**, the voltage at which half the channels were inactivated was found to be approximately -19 mV, with a slope (k) of 4.3 (**Figure 4**). This is again in agreement with Rae and Shepard, 2000; Fernandez *et al.*, 2003 ($V_{½}$ of inactivation = -22.5 to -12 mV and k = 6.7).

Figure 4. Inactivation of the hKv3.3 current. Cells were stepped from –100 mV to +50 mV for 2 s from the holding potential (-80 mV) in 10 mV increments (conditioning voltage) and then stepped to a test voltage of +50 mV for 25 ms to measure channel availability after each conditioning voltage step. Sweeps every 15 s. The current amplitudes, measured at the end of the test voltage step to +50 mV, were normalized to current obtained after the 2 s step to -100 mV. This is plotted against the conditioning voltage and could be described by a Boltzmann equation. Inactivation $V_{½}$ = -18.5 ± 1.9 mV and slope (k) = 4.3 ± 0.2 (n = 3).



The hKv3.3 current exhibits significant inactivation during 500 ms voltage steps to positive potentials (example trace **Figure 5**). The time course of this decay could be described by a single exponential with a time constant (τ) of 107 ± 9 ms. These values are in close agreement with the findings of Fernandez *et al*. 2003 (τ = 92 ms but note that this data was obtained from the teleost homologue of the Kv3.3 channel expressed in CHO cells).

Figure 5. Rate of inactivation the hKv3.3 channel.
Typical current trace of hKv3.3 channel stepped to +30 mV from a holding potential of –80 mV for 200 ms. Time course of inactivation (τ) = 107 ± 9ms, mean ± SEM (n = 4).

Pharmacology - 4-Aminopyridine:
hKv3.3 currents were inhibited by micromolar concentrations 4-Aminopyridine (4-AP) - see **Figure 6**. The application of 4-AP (60 µM) was found to inhibit the hKv3.3 current by >70 % (**Table 1**). 4-AP has a reported IC_{50} of <100 µM on Kv3.3 currents when expressed in mammalian cells (Rashid *et al.*, 2001). Again, note that these findings were obtained using the teleost homologue of Kv3.3.

Figure 6. Effect of 4-AP on hKv3.3 currents.
A. Current traces evoked by stepping to 0 mV for 200 ms from a holding potential of –80 mV, before (black trace), in the presence of (blue), and after wash off (grey) of 60 µM 4-AP. Scale bars represent 50 ms and 500 pA.
B. Typical time course of inhibition of hKv3.3 currents by 60 µM 4-AP. Currents were evoked by stepping to 0 mV for 200 ms from -80 mV. Sweeps every 10 s. Currents normalized to current before the addition of 4-AP.

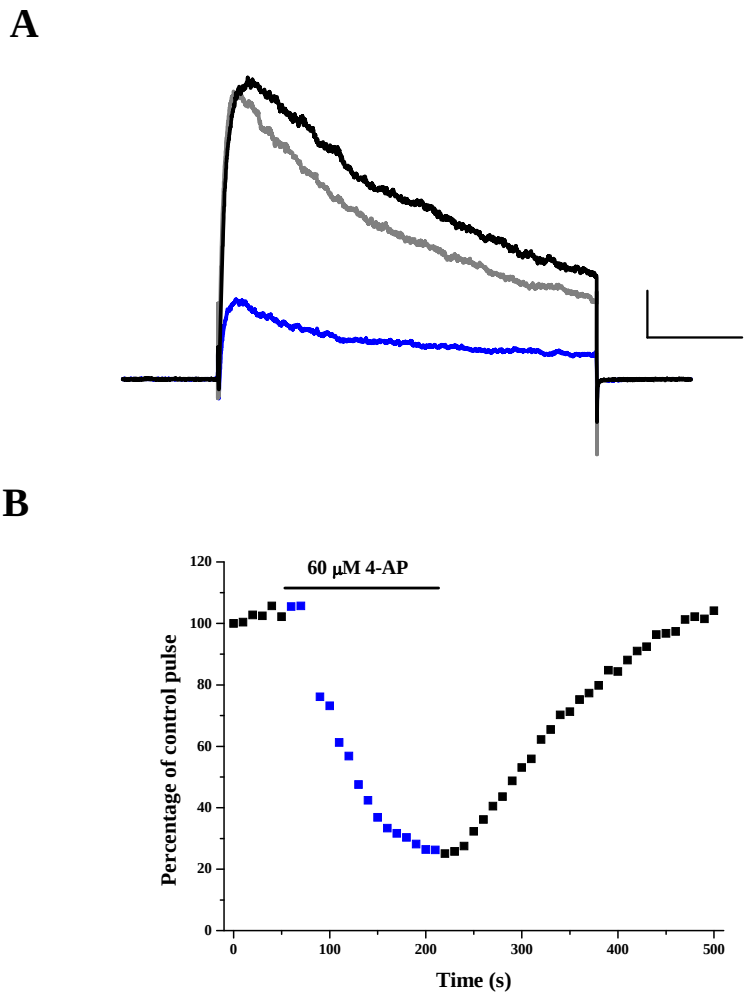


Table 1. Inhibition of hKv3.3 currents by 60 µM 4-AP.

Percentage inhibition of control current (mean ± SEM)	Number of cells
75.7 ± 6.2	4

Pharmacology – Tetraethylammonium chloride:
140 µM Tetraethylammonium chloride (TEA) inhibited hKv3.3 currents by approximately 50% (**Figure 7** and **Table 2**). This is in agreement with the published IC_{50} of 140 µM (Vega-Saenz de Miera *et al.*, 1992).

Figure 7. The effect of TEA on hKv3.3 currents (clone 13).
A. Current traces evoked by stepping to 0 mV from a holding potential of –80 mV before (black trace), in the presence (green trace) and after wash off (grey trace) of 140 µM TEA. Scale bars represent 50 ms and 500 pA.
B. Typical time course of inhibition of hKv3.3 currents by 140 µM TEA. Currents were evoked by stepping to 0 mV for 200 ms from -80 mV. Sweeps every 10 s. Currents normalized to current before the addition of TEA.

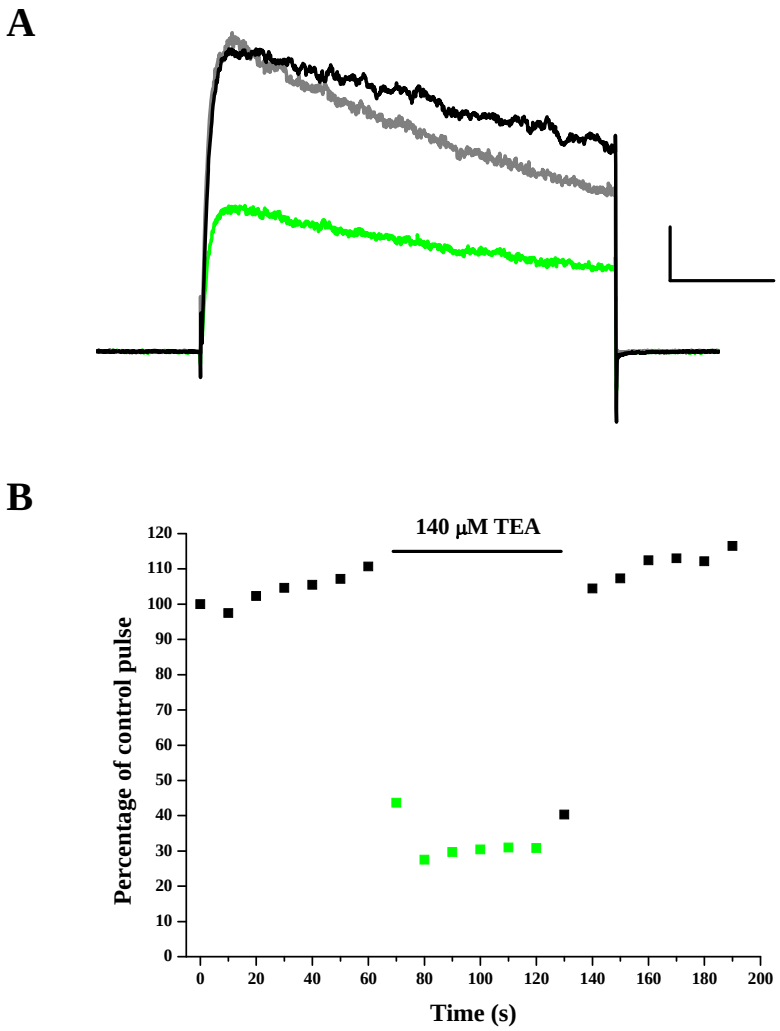


Table 2. Inhibition of hKv3.3 currents by 140 µM TEA.

Percentage inhibition of control current (mean ± SEM)	Number of cells
57.5 ± 4.6	8

Stability of hKv3.3-CHO K1 Cell Line.

IonWorks™ HT Electrophysiology.

The hKv3.3-CHO K1 cell line has stable expression for > 37 passages.

Functional channel expression, defined as cells expressing hKv3.3 current of ≥ 500pA, was monitored using IonWorks™ HT. This data and the mean current amplitude is shown in **Figure 8**. Number of cells expressing a current ≥ 500 pA shown above mean current amplitude data. Sealing data is shown in **Figure 10**.

Figure 8. Stability of expression over passage.
The upper panel shows the percentage of cells expressing a mean peak tail current >500 pA at cell passages 1, 10, 16, 26, 31 and 37. The lower panel shows the mean current amplitude (mean ± SEM, red circles) and the number of these cells (numbers above red circles).

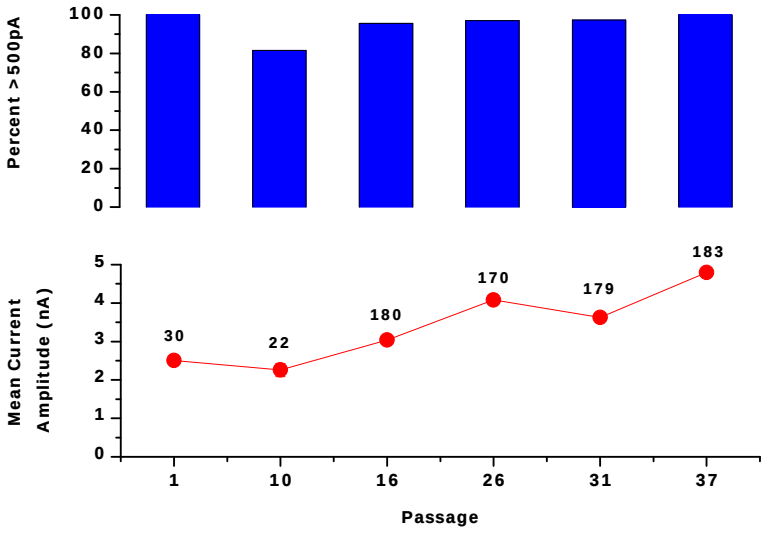
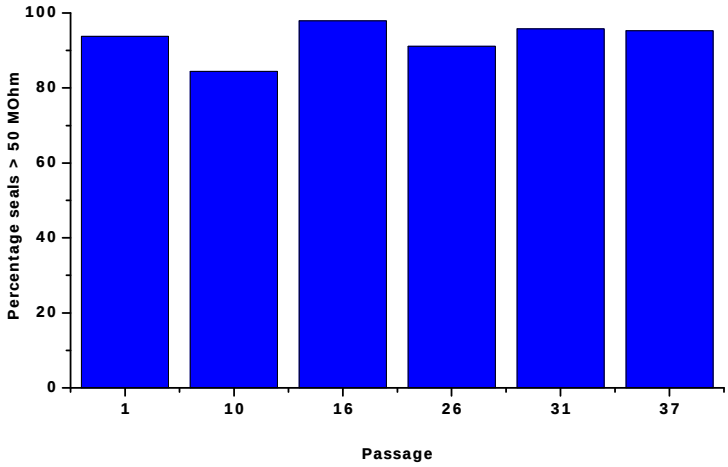


Figure 10. Sealing rates over passage.
The percentage of cells sealing (defined by a seal resistance of >50 MOhm).



Recommended Culture Conditions:

Cells should be grown in a humidified environment at 37°C under 5% CO_2 using F-12 Nutrient Mixture (Ham) + Glutamax medium supplemented with 10% Foetal Bovine Serum plus 400 µg/ml of Geneticin to ensure that the recombinant expression is maintained.

Transfection of CHO-K1 host cells with the human Kv3.3-pCIN5 construct does not appear to have retarded the growth characteristic of the host cells, which exhibit a normal cell division time of approximately 16 hours.

It is recommended to rapidly thaw a frozen aliquot from liquid nitrogen by agitation in a 37°C water-bath and to transfer vial contents into a T175 cm^2 flask containing 50 ml of pre-equilibrated media according to the formulation below. Allow cells to adhere for 4-8 hours at 37°C under 5% CO_2 before gently removing the media and replacing with 30 ml of fresh media.

The cell line should not be allowed to exceed 80% confluency within the culture vessel, to prevent contact inhibition causing senescence. Passage every 2-3 days by rinsing with phosphate buffered saline before harvesting with Trypsin/EDTA and seeding into new flasks using a seeding density of 0.5-1×10^6 cells per T75 cm^2 or 1-2×10^6 cells per T175 cm^2 flask. It is essential that the cell line is continually maintained in the presence of Geneticin (400 µg/ml), which should be added to the culture vessel or media immediately prior to use.

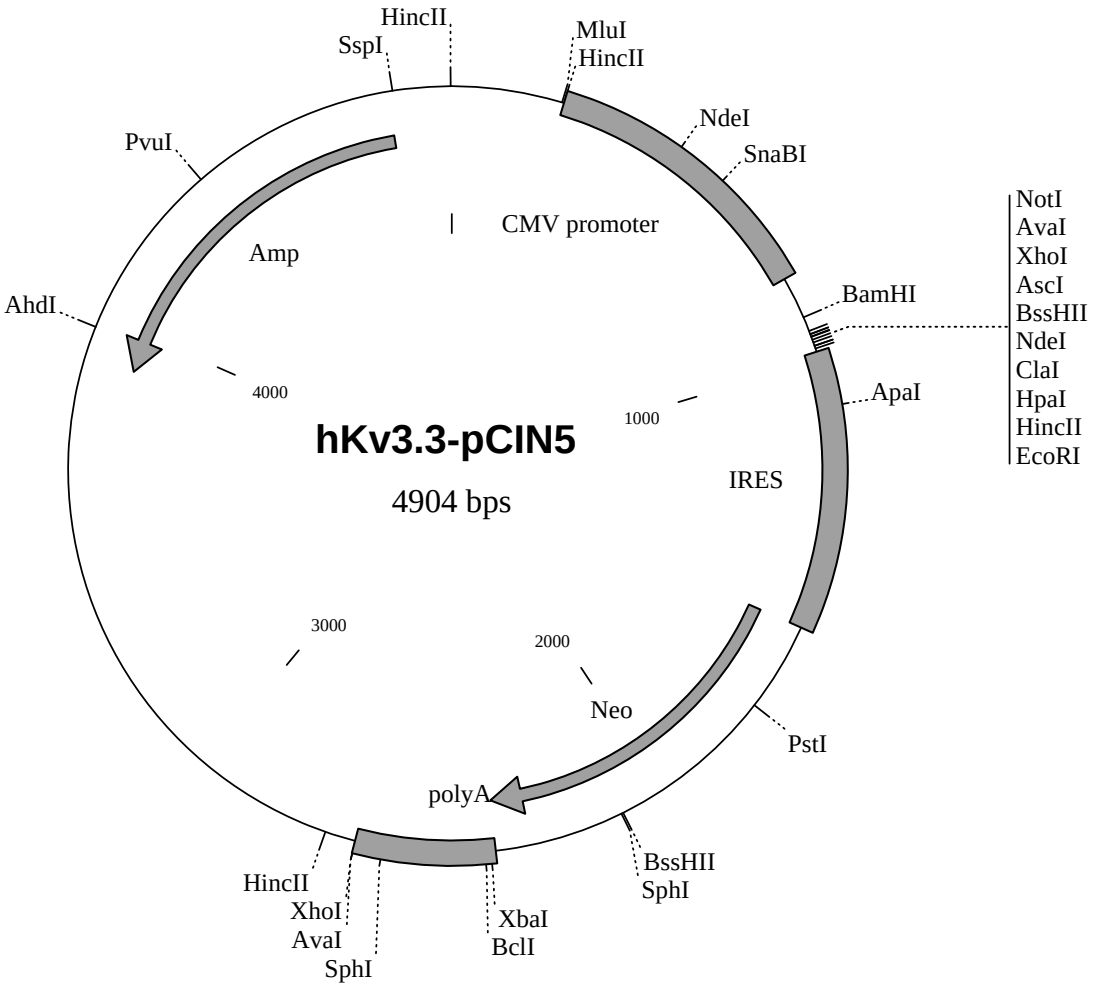
Media Formulation:

F-12 Nutrient Mixture (Ham) (with GlutaMAX™ I)	(Invitrogen	#31765)
10% Foetal Bovine Serum	(Invitrogen	#16000)
400 µg/ml Geneticin	(Invitrogen	#10131)

Other reagents required:

Trypsin/EDTA	(Invitrogen	#25300)
PBS	(Invitrogen	#14190)
Trypan Blue	(Sigma	#T8154)
DMSO	(Sigma	#D2650)

Vector:



Polylinker: CMV-BamHI-NotI-**hKv3.3**-EcoRI-IRES-*neo*

hKv3.3 Sequence:

Bases 434-2274 of sequence of the cDNA used to make this cell line correspond to the accession number NM_004977. Bases 1-433 were constructed synthetically with altered codon usage.

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